# NITRITE METABOLISM BY MUSCLE *IN VITRO*

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## SUMMARY

1. The anaerobic incubation of minces of fresh pig muscles at pH 6.0 with sodium nitrite, but not with potassium nitrate, resulted in the evolution of a gas fraction which was insoluble in alkali but was soluble in alkaline sulphite, corresponding in solubility properties to nitric oxide.

2. Nitric oxide was identified as a product of the anaerobic incubation of muscle minces with sodium nitrite at pH 6.0 by mass spectrometry and infrared spectrophotometry; its presence was also recognised by a new technique involving the increased absorbancy in the ultraviolet of the alkaline sulphite absorption liquors and by the conversion of indigenous haem pigment to nitrosylmyoglobin.

3. The output of the nitric oxide gas fraction was unaffected by the inclusion of the antibiotics aureomycin and chloromycetin and neither the aerobic nor the anaerobic development of the small resident bacterial populations of fresh muscle minces resulted in the enhancement of their capacities to reduce nitrite to nitric oxide, whereas the reduction of nitrate was apparent only with tissues in which bacterial growth had been deliberately encouraged.

4. The formation of the nitric oxide gas fraction was markedly reduced by heat treatment of the minces at 56° and 80°, was dependent upon nitrite concentration and was stimulated by reduced methylene blue; this and other evidence indicates that nitrite is able to compete with other electron acceptors for the reductive processes of the mammalian respiratory enzyme systems.

No direct interaction at pH 6.0 was observed between $NaNO_2$ and Hb, Mb, GSH and ferrocytochrome *c*.

## INTRODUCTION

A number of enzyme systems has been characterized in bacteria, fungi and green plants which catalyze the reduction of nitrate to ammonia via nitrite and hydroxylamine[1], and nitric oxide has been recognised as the product of the action of a nitrite reductase system from *Pseudomonas aeruginosa*[2]. In addition, an enzyme system capable of reducing nitrate to nitrite, hyponitrite, hydroxylamine and ammonia has been reported in hen liver[3], but the ability of mammalian tissues to effect the reduction of nitrite has not so far been recognised, although the oxidation–reduction

Abbreviations: NOMb, nitrosylmyoglobin; NOHb, nitrosylhaemoglobin.

potential for the change: $HNO_2 + H^+ + e \rightleftharpoons NO + H_2O$ is $+ 0.99$ V (ref. 4), so that the system is not precluded electronically from providing a ready acceptor for the electrons of the mammalian respiratory chain normally directed aerobically towards oxygen.

Evidence is presented herein for small capacities of fresh muscle minces to reduce sodium nitrite anaerobically to nitric oxide under conditions such that the action has been attributed to mammalian enzyme systems rather than to those of contaminating bacteria.

## METHODS

### Manometry

Muscle tissue, obtained from local slaughterers or from the National Pig Progeny Testing Board, Stotfold, Bedfordshire, and generally used within 24–48 h of death, was minced in a small tissue grinder (A. Gallenkamp & Co., London). Samples of minces (usually 3.0 g) were placed in Warburg flasks with 3.0 ml 0.20 M phosphate buffer (pH 6.0) containing antibiotics where stated; absorbents were either of water, 0.9 N KOH or alkaline sodium sulphite[5]. After equilibration at 37° with argon passing through the flasks, $NaNO_2$ or $KNO_3$ (usually 0.5 ml of a 3.0 % soln.) was tipped in from the side arm; for the secondary inclusion of $NaNO_3$ subsequent to incubation with $KNO_3$, a 3.0 % soln., bubbled with argon to remove dissolved oxygen, was injected down the gas port leading to the side arm, whence it was sucked in by temporary removal of the flask from the water bath. Methylene blue soln. (0.37 mg/ml), after reduction with Pt-black and $H_2$, was introduced anaerobically into Warburg flasks through which argon was flowing with little reoxidation of the dye.

### Mass spectrometry

Pig muscles were minced 2 h after death; fresh minces (4.0 g) were placed in Thunberg tubes with 5.0 ml 0.20 M phosphate buffer (pH 6.0) containing chloromycetin (10 mg%) with 1.0 ml 3.0 % $NaNO_2$ in the bulbs. The tubes were evacuated and filled repeatedly with argon pretreated in a Nilox apparatus (Southern Analytical Ltd., Camberley, Surrey) to remove any traces of oxygen, the final atmosphere being one of argon at reduced pressure. After mixing, an incubation period of 2 h at 37° was allowed; the gaseous products of the incubations were transferred under high vacuum into an A.E.I. MS3 mass spectrometer.

### Infrared absorbancy studies

Incubations were conducted in Drechsel bottles fitted with high vacuum stopcocks using 100-g samples of muscle mince in 35 ml 0.20 M phosphate buffer (pH 6.0) containing chloromycetin (10 mg%); 10 ml 3.0 % $NaNO_2$ or $KNO_3$ were contained in test tubes wedged in the minces. Repeated evacuation and filling with argon to produce a final atmosphere of argon under reduced pressure preceded admixture and incubation for 2 h at 37°. The gaseous products were transferred over $P_2O_5$ through glass tubing, which had been thoroughly flushed with argon, into an evacuated gas cell (NaCl plates, path length 6 cm) for spectral observations in a Unicam SP 100 spectrophotometer.

Absorbancies in the visible and ultraviolet regions were determined in a Unicam SP 500 spectrophotometer using cells of 1 cm light path.

### Nitrosylmyoglobin estimation

MetMb was isolated from pig hearts[6] and from it were derived Mb, $MbO_2$ and NOMb by treatment in aqueous solution with a small amount of $Na_2S_2O_4$ followed by $O_2$ or $NaNO_2$. Of the four pigments, only NOMb yielded material absorbing at 540 m$\mu$ upon dropwise addition with shaking of acetone to a concn. of 80 % (v/v), the solution having the spectrum of a nitrosylhaemochromogen[7] with a millimolar absorbancy coefficient at 540 m$\mu$ of 7.0 on a basis of one atom of Fe per molecule. NOMb contents of incubation products were determined at this wavelength by the dropwise addition of acetone with shaking to a concn. of 80 %, including allowance for water bound by the muscle minces, and filtration away from direct light.

### Bacteriology*

For aerobic counts decimal dilutions of tissue homogenates (3.0 g in 145 ml 0.9 % NaCl) were plated on "Oxoid" Plate Count Agar (Oxo Ltd., London); the plates were incubated for 24 h at 37° before counting.

For anaerobic use 5.0 g muscle mince was homogenized in 100 ml distilled water, from which concn. decimal dilutions were made in distilled water. Surface counts[8] were prepared from appropriate dilutions on 5 % horse-blood agar with anaerobic incubation for 24 h at 37° in a McIntosh and Fildes' jar.

Nitrite determinations were undertaken by a modification of the method of SNELL AND SNELL[9].

### Reducing agents

$NADH_2$ and cytochrome $c$ (Fe = 0.35 %) were supplied by L. Light & Co., Colnbrook, Bucks.; spectrophotometric estimations of the former were undertaken at 340 m$\mu$ (ref. 10) and of the latter, after reduction with $H_2$/Pt black, at 550 m$\mu$ (ref. 11). Aureomycin was obtained from Cyanamid Products Ltd., London, and chloromycetin from Parke Davis and Co., Hounslow.

<div align="center">RESULTS</div>

### Gas exchange experiments

Fig. 1 illustrates gas exchanges obtained upon the anaerobic incubation of minces of pig skeletal muscles with $NaNO_2$ at pH 6.0; such gas evolutions were not observed in the absence of either tissue or nitrite. Part of the gas evolved was absorbed by 0.9 N KOH, a further part was soluble in alkaline sodium sulphite[5], the small remainder being insoluble in both absorbents.

The production of the gas component soluble in alkaline sulphite but not in alkali (corresponding to the solubility properties of NO) was not reduced by the inclusion of the broad-spectrum antibiotics aureomycin and chloromycetin (Table I). The latter, chosen as of greater solubility and broader spectrum of action[12], was included in all subsequent incubations as a bacteriostatic agent. Under these conditions, more than 100 samples of pig muscle (mainly the semimembranosus, biceps femoris and quadriceps femoris) have yielded NO gas fractions of 0–2.0 $\mu$l/min with an average of the order of 0.8 $\mu$l/min. The capacity of pig muscle minces to produce this gas

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* Undertaken by the Bacteriological Department, B.F.M.I.R.A., Leatherhead.

fraction was markedly reduced by treatment of the tissues in buffer at 56° and 80° for 10 min (Table II).

The addition of $NaNO_2$ to muscle minces incubated anaerobically at pH 6.0 after the cessation of the small endogenous respiration in the absence of oxygen produced an immediate evolution of gas, most of which was not absorbed by 0.9 N
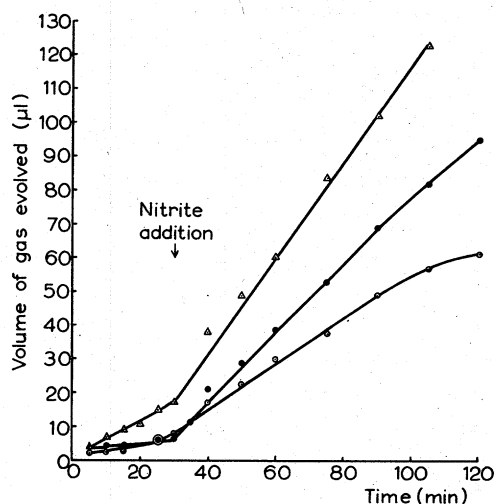


Fig. 1. Gas exchanges resulting from the anaerobic incubation of $NaNO_2$ with pig-muscle mince at pH 6.0 in the presence of water (△—△), 0.9 N KOH (●—●) and alkaline sodium sulphite (⊙—⊙) as absorbents. 3.0-g samples of tissue suspended in 3.0 ml 0.20 M phosphate buffer (pH 6.0) under argon; additions of $NaNO_2$ to 62 mM made at the time indicated.

KOH (Fig. 2). The immediate inclusion of $NaNO_2$ into identical systems resulted in an increased overall respiration in comparison with nitrite-free incubations. In contrast to these anaerobic effects, the addition of $NaNO_2$ to muscle minces in air under otherwise identical conditions depressed their overall endogenous respirations, the concentration required for 50 % inhibition being of the order of 50 mM.

Under anaerobic conditions, the incubation of similar minces with 43 mM $KNO_3$ did not result in the evolution of significant gas, although the later inclusion of

## TABLE I

EFFECT OF BROAD-SPECTRUM ANTIBIOTICS ON GAS EVOLUTION FROM $NaNO_2$
INCUBATED WITH MUSCLE MINCES AT pH 6.0

Incubations under argon of 3.0-g samples of muscle minces in 3.0 ml 0.20 M phosphate buffer (pH 6.0) containing antibiotic (10 mg%), with 0.5 ml 3.0 % $NaNO_2$ initially in side arms; 0.9 N KOH and alkaline sodium sulphite[5] as absorbents.

| Antibiotic | Average evolution of gas without antibiotic (µl/min) | | | Average evolution of gas in presence of antibiotic (µl/min) | | |
|---|---|---|---|---|---|---|
| | Alkali absorbent | Alkaline sulphite absorbent | Difference | Alkali absorbent | Alkaline sulphite absorbent | Difference |
| Aureomycin | 0.81 | 0.40 | 0.41 | 0.93 | 0.52 | 0.41 |
| Chloromycetin | 0.52 | 0.21 | 0.31 | 0.60 | 0.30 | 0.30 |

TABLE II

EFFECT OF HEAT TREATMENT ON GAS EVOLUTION FROM $NaNO_2$ INCUBATED
WITH MUSCLE MINCES AT pH 6.0

Incubations under argon of 3.0-g samples of muscle minces with and without pretreatment for
10 min in a water bath at the temperature indicated in 0.20 M phosphate buffer (pH 6.0) containing
chloromycetin (10 mg%), with 0.5 ml 3.0% $NaNO_2$ initially in side arms; 0.9 N KOH and alkaline
sodium sulphite[5] as absorbents.

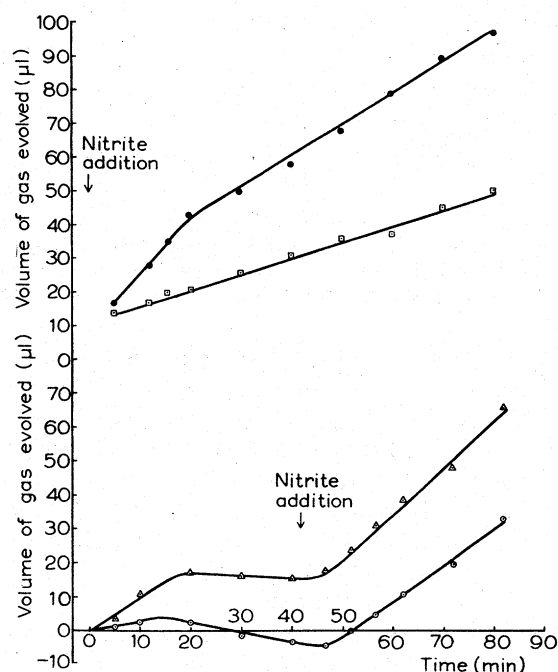| Temperature of heat treatment | Average evolution of gas without heat treatment ($\mu l/min$) | | | Average evolution of gas after heat treatment ($\mu l/min$) | | |
|---|---|---|---|---|---|---|
| | Alkali absorbent | Alkaline sulphite absorbent | Difference | Alkali absorbent | Alkaline sulphite absorbent | Difference |
| 56° | 0.45 | 0.19 | 0.26 | 0.25 | 0.16 | 0.09 |
| 56° | 0.57 | 0.25 | 0.32 | 0.43 | 0.34 | 0.09 |
| 80° | 0.39 | 0.14 | 0.25 | 0.21 | 0.12 | 0.09 |
| 80° | 0.50 | 0.24 | 0.25 | 0.12 | 0.11 | 0.01 |



Fig. 2. Gas exchanges resulting from the inclusion of $NaNO_2$ during the anaerobic incubation of a
pig-muscle mince at pH 6.0 in the presence of chloromycetin, with water (●—●) and △—△),
0.9 N KOH (⊙—⊙) and alkaline sodium sulphite (□—□) as absorbents. 3.0-g samples of tissue
suspended in 3.0 ml 0.20 M phosphate buffer (pH 6.0) containing chloromycetin (10 mg) under
argon; additions of $NaNO_2$ to 62 mM made at the times indicated

$NaNO_2$ (Fig. 3) into systems already incubated with nitrate provided immediate
evidence of their capacities to evolve gas in the presence of nitrite.

*Gas identification studies*

No nitrogen was detected by the micro-Kjeldahl technique in the alkaline
sulphite absorption liquors from the incubation of minces with $NaNO_2$; however, the

formation of $NH_3$ after reduction with Devarda's alloy in Conway units of the absorbents from anaerobic incubations was detected by the phenol–hypochlorite method[13].

Facilities for mass spectrometry and an interpretation of the results were provided through the courtesy of the British Petroleum Co., Sunbury-on-Thames, Great Britain. A peak at mass/charge ratio of 30, attributed to NO, was observed with the gaseous products of anaerobic incubation at pH 6.0 with $NaNO_2$ of minces of fresh pig gastrocnemius, soleus and external digitalis muscles, the reported NO content varying between 9 and 27 % on a water-vapour-free basis whilst a control mixture without mince contained only 0.5 %. $N_2$ was also present and in all cases a peak was observed at 44. The appearance in the mass spectrometer of a peak at this mass/charge ratio due to single-charged $CO_2$ molecules is accompanied by a small peak at 22 corresponding to doubly-charged $CO_2$ molecules. In four out of five experiments the small secondary peak was absent and the peak at 44 was therefore ascribed completely to $N_2O$.

Through the cooperation of Mr. R. BENT and Dr. W. R. LADNER of the British Coal Utilisation Research Association, Leatherhead, Great Britain, the infrared spectra of the dried gaseous products of the anaerobic incubation of minces of pig muscles with $NaNO_2$ or $KNO_3$ at pH 6.0 were examined at 3000–650 $cm^{-1}$. Any
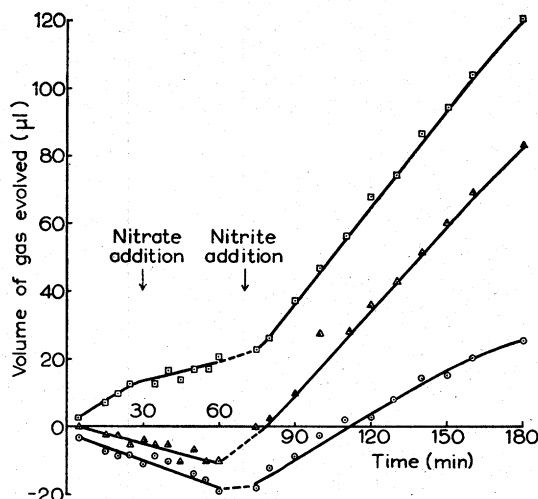


Fig. 3. Gas exchanges resulting from the anaerobic incubation of firstly $KNO_3$ and secondly $NaNO_2$ with a pig-muscle mince at pH 6.0 in the presence of chloromycetin with water (□—□), 0.9 N KOH (△—△) and alkaline sodium sulphite (⊙—⊙) as absorbents. 3.0-g samples of tissue suspended in 3.0 ml 0.20 M phosphate buffer (pH 6.0) containing chloromycetin (10 mg%) under argon; additions of $KNO_3$ to 43 mM and of $NaNO_2$ to approx. 55 mM made at the times indicated.

small peak at 1870 $cm^{-1}$ due to NO could not be recognised with certainty over and above the background absorption ascribed to small amounts of residual water vapour. The admission of oxygen, however, resulted in a large increase in intensity at 1615 $cm^{-1}$ in all tests involving nitrite, in accordance with the conversion of the weak absorber NO to the strong absorber $NO_2$, with its main peak at this wavelength. No NO could be detected in this way from digests of buffer and $NaNO_2$ without mince,

or of mince and buffer without $NaNO_2$. Incubation with $KNO_3$ of minces active towards $NaNO_2$ also failed to yield NO detectable as $NO_2$.

In addition, the formation of NO during the anaerobic incubation of muscle minces with $NaNO_2$ at pH 6.0 was detected by the increases in absorbancy in the ultraviolet of the alkali and alkaline sulphite absorption reagents[14] employed in the Warburg flasks. Other soluble gases possibly formed during the utilization of $NaNO_2$ by muscle minces have not been found to increase the absorbancy of the alkaline sulphite reagent appreciably.

*Bacterial development and reduction of nitrite and nitrate*

The incubation of pig muscle minces at 25° for 24 h in air with consequent enhancement of the aerobic microbial flora did not lead to an increase in their capacities to effect anaerobic reduction of $NaNO_2$ at pH 6.0 to a NO gas fraction (Table III). Further samples of the same tissues maintained at —20° for 48 h before mincing retained at least in part their capacities in this respect. After aerobic incubation at 25° for 24 h, muscle minces acquired activity towards $KNO_3$ in some cases with the production of nitrite and/or a NO gas fraction.

TABLE III

PRODUCTION OF NITRIC OXIDE GAS FRACTION FROM THE ANAEROBIC INCUBATION AT pH 6.0 OF
$NaNO_2$ WITH FRESH MUSCLE MINCES AND THOSE PREVIOUSLY INCUBATED TO ENCOURAGE AEROBIC
BACTERIAL GROWTH

Incubations under argon of 3.0-g samples of muscle minces in 3.0 ml 0.20 M phosphate buffer (pH 6.0) containing chloromycetin (10 mg%) with 0.5 ml 3.0% $NaNO_2$ initially in side arms.

| Sample | Tissue preparation | Aerobic plate count (number per g) | Evolution of nitric oxide gas fraction ($\mu l/min$) |
|--------|--------------------|-----------------------------------|------------------------------------------------------|
| A | Fresh muscle mince | $1.1 \cdot 10^5$ | 1.1 |
| | Mince incubated at 25° for 24 h | $1.8 \cdot 10^8$ | 0.3 |
| | Muscle minced after maintenance at —20° for 48 h | — | 0.32 |
| B | Fresh muscle mince | $4.0 \cdot 10^4$ | 0.40 |
| | Mince incubated at 25° for 24 h | $1.2 \cdot 10^8$ | 0.0 |
| | Muscle minced after maintenance at —20° for 48 h | $5.6 \cdot 10^4$ | 0.34 |
| C | Fresh muscle mince | $6.0 \cdot 10^4$ | 0.30 |
| | Mince incubated at 35° for 24 h | $2.0 \cdot 10^8$ | 0.0 |
| | Muscle minced after maintenance at —20° for 48 h | $1.0 \cdot 10^5$ | 0.31 |

In studying the effects of the multiplication of obligate as well as facultative anaerobes on $NaNO_2$ reduction, the percentage conversion of indigenous haem pigment to NOMb was used as a more sensitive criterion of NO formation. Minces of fresh muscle of low anaerobic bacterial count were again consistently able to metabolize $NaNO_2$ but not $KNO_3$ on anaerobic incubation at pH 6.0. After anaerobic incubation of the minces at 30° for 18 h, their capacities to form NOMb from $NaNO_2$ were virtually unchanged, whilst an appreciable formation of NO from $KNO_3$ was apparent in all instances (Table IV). Other samples of the same muscle tissues were maintained frozen at —20° for 48 h before mincing; these minces retained their abilities to reduce $NaNO_2$ at least in part but were inactive towards $KNO_3$.

## TABLE IV

PRODUCTION OF NOMb FROM $NaNO_2$ AND $KNO_3$ AFTER ANAEROBIC INCUBATION AT pH 6.0
WITH FRESH MUSCLE MINCES AND THOSE PREVIOUSLY INCUBATED TO
ENCOURAGE ANAEROBIC BACTERIAL GROWTH

Incubations under argon for 2 h of 3.0-g samples of muscle minces in 3.0 ml 0.20 M phosphate buffer (pH 6.0) containing chloromycetin (10 mg%) with 0.5 ml of either 0.12% $NaNO_2$ or 0.17% $KNO_3$ initially in side arms of Thunberg tubes.

| Sample | Tissue preparation | Anaerobic plate count (number per g) | Percentage conversion of indigenous pigment to nitrosylmyoglobin in presence of | |
|---|---|---|---|---|
| | | | NaNO₂ | KNO₃ |
| A | Fresh muscle mince | $9.5 \cdot 10^3$ | 71 | 1.4 |
| | Mince incubated anaerobically at 30° for 18 h | $4.9 \cdot 10^8$ | 76 | 63 |
| B | Fresh muscle mince | $2.1 \cdot 10^3$ | 71 | 1.8 |
| | Mince incubated anaerobically at 30° for 18 h | $2.1 \cdot 10^8$ | 70 | 58 |
| | Muscle minced after maintenance at —20° for 48 h | — | 64 | 5.6 |
| C | Fresh muscle mince | $4.7 \cdot 10^3$ | 70 | 0.0 |
| | Mince incubated anaerobically at 30° for 18 h | $1.3 \cdot 10^9$ | 79 | 71 |
| | Muscle minced after maintenance at —20° for 48 h | $4.0 \cdot 10^6$ | 77 | 0.0 |

### Attempts at a stoichiometric relationship

The evolution of the alkali-insoluble and alkaline-sulphite-soluble gas fractions was dependent upon the nitrite concentration, the output with 62 mM $NaNO_2$ being reduced to about one-third on lowering the concentration to 20 mM. The NO gas fraction was very much reduced and sometimes eliminated when the nitrite concentration was lowered to 0.72 mM in attempts to obtain complete reduction. However, the absorbancies in the ultraviolet of the two absorption liquors were always increased, indicating that small amounts of NO had been absorbed by both. In studying the metabolic fate of $NaNO_2$ on incubation with muscle minces the amounts of free NO were estimated from the increases in absorbancy of the alkaline sulphite liquors at 270 m$\mu$ (ref. 14). As an example, after incubations of 170 $\mu$g $NaNO_2$ with 3.0-g samples of a muscle mince under the usual conditions for 90 min, an average of 44 $\mu$g of the salt remained unchanged in solution whilst NO equivalent to 8.4 $\mu$g and 98 $\mu$g $NaNO_2$ was located spectrophotometrically in combination with muscle Mb as NOMb and in solution in the alkaline sulphite absorbent, respectively.

NOMb formation on incubation of muscle minces with 62 mM $NaNO_2$ in air for 90 min was only 34–42% of that produced under argon.

### Nitrite reduction and its relation to cellular components

Any possibility of the implication in nitrite reduction of contaminating serum enzymes or Hb directly was removed by the failure to obtain NO evolution on the anaerobic incubation of $NaNO_2$ (48 mM) with whole citrated pig bloods at pH 6.0. At the same time, $HbO_2$ present was oxidised to MetHb without concurrent formation of NO; purified pig heart Mb (ref. 6) similarly does not reduce nitrite to NO.

$NADH_2$ has been reported to reduce nitrite under anaerobic conditions at pH 4.0 to NO, $N_2O$ and $N_2$ (ref. 15); at pH 6.0, however, no direct non-enzymic interaction was observed between the two compounds in equimolecular proportions,

provided that the reduced nucleotide was removed by precipitation with barium acetate and ethanol[16] before the estimation of residual nitrite was done. GSH, which has been reported to reduce nitrite rapidly at pH 3.0 to $NH_2OH$ and $NH_3$ (ref. 17), did not produce any NO on anaerobic incubation of 16 $\mu$moles with excess nitrite at pH 6.0.

The reduction of nitrite by ascorbate at pH 3–5 to NO, $N_2O$ and $N_2$ was virtually eliminated at pH 6 according to EVANS AND McAULIFFE[15] using an ascorbate : nitrite molar ratio of 100 : 1. Nevertheless, the anaerobic incubation of 8.6 mM sodium ascorbate with 62 mM $NaNO_2$ at pH 6.0 was found manometrically to produce an appreciable NO fraction, the identity of which was confirmed by a concurrent increase in absorbancy at 270 m$\mu$ of the alkaline sulphite absorbents; fresh muscle minces, however, were not found to contain appreciable amounts of ascorbic acid.

TABLE V

EFFECTS OF REDUCED METHYLENE BLUE ON GAS EVOLUTION FROM THE ANAEROBIC INCUBATION OF $NaNO_2$ WITH FRESH MUSCLE MINCES

Incubations under argon of 3.0-g samples of muscle minces in 3.0 ml 0.20 M phosphate buffer (pH 6.0) containing chloromycetin (10 mg%) with 0.5 ml 3.0% $NaNO_2$ initially in side arms; reduced methylene blue included anaerobically to concn. of 0.024 mg/ml. Absorbents of 0.9 N KOH and alkaline sodium sulphite[5].

| Average evolution of gas without reduced methylene blue in presence of ($\mu l/min$) | | | Average evolution of gas with reduced methylene blue in presence of ($\mu l/min$) | | |
|---|---|---|---|---|---|
| Alkali absorbent | Alkaline sulphite absorbent | Evolution of nitric oxide gas fraction | Alkali absorbent | Alkaline sulphite absorbent | Evolution of nitric oxide gas fraction |
| 0.72 | 0.45 | 0.27 | 1.4 | 0.71 | 0.70 |
| 0.79 | 0.39 | 0.40 | 1.2 | 0.60 | 0.60 |
| 1.2 | 0.72 | 0.48 | 1.4 | 0.63 | 0.77 |

The electron donor reduced methylene blue ($E_0' = + 0.01$ V, pH 7.0, 30°) provoked almost consistently a stimulation of the NO gas fraction obtained upon the incubation of fresh muscle minces with $NaNO_2$ at pH 6.0 (Table V), although no gas evolution resulted from the direct interaction of the reduced dye and $NaNO_2$ at identical concentrations. This fact prompted interest in the components of the respiratory chain with oxidation–reduction potentials intermediate between that of methylene blue and that of the nitrite–nitric oxide systems and in particular towards cytochrome $c$; ferrocytochrome $c$, however, was not oxidised directly by $NaNO_2$.

DISCUSSION

The persistence of the capacity of a large number of muscle minces to effect anaerobic reduction of $NaNO_2$ in the presence of broad-spectrum antibiotics, together with the failure of the development of the bacterial populations of the minces under both aerobic and anaerobic conditions to enhance nitrite reduction, provide strong evidence that the action was not dependent upon bacterial contamination.

In contrast, the requirement of the intervention of bacteria for the utilization of $KNO_3$ by muscle minces is readily apparent. The inability of fresh minces to accom-

plish a closely related biochemical change is yet another indication of the participation of the mammalian systems *per se* in the reduction of nitrite; all of the routes of the bacterial reduction of nitrite discussed by TANIGUCHI, SATO AND EGAMI[18], for instance, involve the prior reduction of nitrate to nitrite, although the enzyme systems responsible for the various stages are presumably different in that they can be partially separated.

Sodium and potassium $N$-nitrosohydroxylamine-$N$-sulphonates, the products of interaction of NO and alkaline sodium sulphite, are decomposed rapidly in acid solution to sulphate and $N_2O$ (ref. 19), which presumably accounts for the failure to detect nitrogen by the micro-Kjeldahl technique in the alkaline sulphite absorption liquors after contact with a NO gas fraction. Nevertheless, NO has been clearly identified as a product of the incubation of fresh muscle minces with $NaNO_2$ under anaerobic conditions at pH 6.0, a value close to the average pH of the minces themselves. The production of gas insoluble in alkaline sulphite in such incubations and the appearance of peaks other than that of NO in mass spectrometry would suggest that reduction can proceed beyond the NO stage if the gas is not removed from the environment, although the $N_2$ peaks detected within the mass spectrometer could be due to breakdown of NO itself.

BROOKS[20] has shown that the reaction of $HbO_2$ and $NaNO_2$ in the absence of oxygen and a reducing agent yields an equimolecular mixture of NOHb and MetHb, whereas MARSHALL AND MARSHALL[21] conclude that NOHb is formed in addition to MetHb only if nitrite is present in sufficient amount. Using pig-heart $MbO_2$ (ref. 6), no evidence of NOMb formation with 0.02 % $NaNO_2$ has been obtained, the only product apparent spectrophotometrically being the MetMb–$NaNO_2$ complex[22]. For the production of NOMb either chemically reducing conditions with, for instance, dithionite or borohydride or biochemical incubation with an active muscle mince is required; similarly, the NO complex of cytochrome $c$ is not formed from the coenzyme and nitrite, but requires the use of gaseous NO. Thus, the conversion of indigenous Mb to its NO complex is considered to provide an index of the formation of the gas.

At low pH values nitrite acts readily and directly as an oxidising agent towards such diverse substances as $NADH_2$ and ascorbic acid[15] and GSH[17], but it is clear from the work of EVANS AND MCAULIFFE[15] that some decomposition occurs at pH 4 even without the intervention of a reducing agent; at pH 6.0, however, no similar instability has been apparent. The maximal reduction of nitrite over a 90-min incubation period produced NO of the order of 0.5 mmoles per 100 g tissue of which approx. 75 % is water-bound or free. A molecular content of this order would thus be required for any muscle component, of necessity heat labile and with an oxidation–reduction potential suitable for reduction by reduced methylene blue, able to reduce nitrite directly without the regeneration of an active reduced form by the muscle enzyme systems, a requirement which is borne out by the observed need for anaerobic conditions.

Evidence for a competition between nitrite and other electron acceptors such as oxygen has been provided by the inhibitory effect of the salt upon endogenous respiration of the minces and the dependence of NO output on nitrite concentration; the decreased NOMb formation in air could also be a manifestation of the same effect but here the outcome is complicated by the possible oxidation of NO in air before combination with haem pigment. However, the affinity of the tissue-respiratory-enzyme

systems for $NaNO_2$ would appear to be much less than that for oxygen in solution judging by the high nitrite concentration required for 50 % inhibition of muscle respiration. The effects of added substrates upon the action were found to be variable and muscle minces undoubtedly represent too heterogeneous an enzyme source for the characterization of the systems involved; however, evidence has already been obtained of a competition between ferricytochrome $c$ and nitrite for the reductive processes of skeletal muscle mitochondria under anaerobic conditions using pyruvate and malate as substrates. The presence of nitrite resulted in a partial inhibition of ferricytochrome $c$ reduction and concurrent formation of its NO complex, and this aspect of the work will be reported more fully later.

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